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Phosphatidylinositol phosphodiesterase of rat brain: Ca^{2+} -dependency, pH optima and heterogeneity

Previous publications from this laboratory have documented the effect of pH, Ca^{2+} , and proteolytic (trypsin) treatment on the soluble PtdIns phosphodiesterase of rat brain (Hirasawa *et al.*, 1982a,b,c). However, examination of the original data from these experiments suggested to us that the characteristics reported should be re-examined under more carefully controlled conditions. Here, we summarize our findings; some of the experimental data will be published elsewhere (Irvine *et al.*, 1984).

PtdIns phosphodiesterase is heterogeneous with respect to its isoelectric point when examined on chromatofocusing columns (Hirasawa *et al.*, 1982a,b). However, we have found that in these experiments the enzyme had run out of substrate at peak levels of activity, so that although the existence and pattern of chromatofocusing peaks reported is correct, their relative magnitudes (Hirasawa *et al.*, 1982a, Fig. 2) are incorrect. Fig. 1(a) is a presentation of the data from one of several such chromatofocusing separations we have since performed on rat brain. The only modifications from the published methods of Hirasawa *et al.* (1982a,b) are: (a) the quantities of fractions assayed were chosen to ensure both a linear reaction rate and utilization of less than 10% of the substrate; (b) the fractions were adjusted with NaOH or HCl to pH 7.25 immediately after collection from the column. This latter precaution was necessary as the pH of the enzyme assay was shifted by up to 1.5 pH units by the strong buffer eluted from the column if the experiments were performed exactly as in Hirasawa *et al.* (1982a,b).

The yields of activities eluted from the column were low (legend to Fig. 1) and any interpretation of relative activities of the various fractions which may exist *in vivo* must be viewed with caution. The activity as pH 5.5 is clearly heterogeneous, however, and in other experiments we could partially resolve the broad peak at pI 5.0–4.4 into two separate peaks. There is only one species showing appreciable activity at neutral pH in the presence of 1 mM- Ca^{2+} . We have not re-examined the liver and kidney chromatofocusing pattern in this respect (cf.

Hirasawa *et al.*, 1982b) but examination of the raw data for those experiments shows that the enzyme had run out of substrate in many assay tubes.

The substrate was also limiting to the enzyme in some tubes represented in Fig. 1 of Hirasawa *et al.* (1982a) (effect of pH on whole brain supernatant at 1 mM- Ca^{2+}). We therefore repeated these experiments and confirmed two pH optima around 5 and 8, but under linear enzyme kinetics the pH 8 activity was much lower than that most active at pH 5. A result of the incorrect pattern published for the control enzyme was the false conclusion that $(\text{NH}_4)_2\text{SO}_4$ precipitation specifically removed the enzyme species active at pH 8. We have repeated this experiment twice and re-examined all the earlier data, and now conclude that there is no evidence for such a specific removal.

In Hirasawa *et al.* (1982c) we reported that the brain supernatant shows little activity at 1 μM - Ca^{2+} , pH 7.25, but the trypsin or autolysis can induce the appearance of such an activity. Although the PtdIns phosphodiesterase reaction is linear with increasing addition of brain supernatant at 1 mM- Ca^{2+} up to high (200 μl of brain supernatant/tube) concentrations, it shows a marked non-linearity when assayed at 1 μM - Ca^{2+} . Consequently, if PtdIns phosphodiesterase is assayed at high enzyme concentrations (100 μl /tube), there is little activity below 0.1 mM and then a sharp rise to 2–3 mM- Ca^{2+} , but if assayed at low (10 μl of enzyme/tube) concentrations the Ca^{2+} -dependency resembles more closely that found in lymphocytes (Allan & Michell, 1974), i.e. maximal at about 1 μM - Ca^{2+} . Furthermore, trypsin treatment removes some of the inhibitory factors operative at high levels of added supernatant (at 1 μM - Ca^{2+}), and consequently partially restores the linearity of the relationship between observed activity and amount of brain supernatant present. A large (200–300%) stimulation is therefore seen after trypsin treatment when 100 μl of supernatant is assayed; this stimulation is undetectable after assay with 10 μl of supernatant. Preincubation of the supernatant alone (Hirasawa *et al.*, 1982c) can also cause a stimulation at 100 μl of enzyme, although over many experiments we have found this to be a very small and inconsistent phenomenon. Even when a small stimulation was observed at 100 μl of enzyme and 1 μM - Ca^{2+} , it was undetectable at lower enzyme concentrations, and we conclude that, like the effect

Abbreviation used: PtdIns, phosphatidylinositol.

Fig. 1. *Chromatofocusing of a rat brain supernatant* (a) Rat brain supernatant (6 ml) was applied to a chromatofocusing column and 6 ml fractions were collected by elution with Polybuffer PB 74 (for details see Hirasawa *et al.*, 1982a,b). Fractions were adjusted to pH approx. 7.25 with HCl or NaOH (and water was added if necessary so that all fractions suffered identical dilution). Samples were taken at this point for all the enzyme assays, and frozen; they were thawed out immediately before assay. Less than 10% of the substrate was hydrolysed in all tubes. $\blacktriangle$, assay at pH 5.5 and 1 mM- Ca^{2+} ; $\circ$, pH 7.0 and 1 mM- Ca^{2+} ; $\bullet$, pH 7.0 and 1 μM - Ca^{2+} (Ca^{2+} /EDTA buffer). The activities under these conditions in the original supernatant applied to the column were 8.9, 0.80 and 1.7 $\mu\text{mol/h}$ per ml respectively, and the recoveries from the column were respectively 24%, 34% and 17%. Reassay of these fractions at pH 8.0 and 1 mM- Ca^{2+} gave a pattern of distribution identical with that at pH 7.0 and 1 mM- Ca^{2+} ($\circ$). (b) A portion (6 ml) of the same supernatant as for (a) (frozen for 3 weeks) was treated with trypsin (120 $\mu\text{g/ml}$) for 20 min, followed by soya bean trypsin inhibitor (100 $\mu\text{g/ml}$). The elution, assay and symbols are exactly as in (a). The trypsin-treated supernatant applied to the column was assayed at pH 5.5 and 1 mM- Ca^{2+} , pH 7.0 and 1 mM- Ca^{2+} and pH 7.0 and 1 μM - Ca^{2+} (Ca^{2+} /EDTA buffer), and contained activities of 7.3, 0.35 and 1.8 $\mu\text{mol/h}$ per ml respectively, recoveries being 8%, 25% and 10% respectively.

of trypsin, it can be entirely ascribed to the removal of inhibitory factor(s) from the supernatant.

Trypsin causes a shift in the isoelectric point of PtdIns phosphodiesterase (Hirasawa *et al.*, 1982c) which we confirm here (Fig. 1b). However, comparison of all peaks of activity at pH 7.25 showed no change in the sensitivity to Ca^{2+} induced by trypsin in any fraction. It is noteworthy that when chromatofocusing fractions are assayed at the correct pH (i.e. with the pH of the fractions corrected to 7.25, and with small amounts of the fractions added to each tube; see above), the activity at 1 μM - Ca^{2+} and pH 7.25 exactly parallels that at 1 mM- Ca^{2+} and pH 5.5 in this (Figs. 1a and 1b) and in two other separate experiments (results not shown). The original brain supernatant shows a pH optimum near 7 when assayed at 1 μM - Ca^{2+} , and the simplest explanation of these various observations is that those enzyme species active at pH 5.5 and 1 mM- Ca^{2+} shift to a higher pH optimum in the absence of any appreciable quantities of cations to balance the charge on the substrate (see Dawson, 1968). Assay of fraction 22 from Fig. 1(a) at (a) 1 mM- Ca^{2+} , (b) 1 μM - Ca^{2+} (Ca^{2+} /EDTA buffers), or (c) 1 μM - Ca^{2+} + 1 mM- Mg^{2+} + 80 mM-KCl (Ca^{2+} /EGTA buffers) showed pH optima at about 5, 6.6 and 5 respectively.

In conclusion, although PtdIns phosphodiesterase is heterogeneous on chromatofocusing, the quantitative details of this heterogeneity (Hirasawa *et al.*, 1982a,b) were incorrect. Furthermore, although proteolysis of the brain supernatant removes some factors inhibitory to the assay at 1 μM - Ca^{2+} , we can find no evidence that such treatment of a brain supernatant causes any change in the Ca^{2+} -sensitivity of the enzyme (Hirasawa *et al.*, 1982c).

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